Cultured Neuron Probe

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General Introduction

Our aim is to develop a cultured neuron probe. This consists of a silicon structure into which individual dissociated neurons can be placed, and which can be inserted into an intact nervous system. Furthermore, within the structure each neuron is in close proximity to an electrode, by means of which it can be stimulated, or its activity can be recorded, through electrical leads which connect to external electronics. It is hoped that neurons in the probe will synaptically integrate with the host nervous system, to provide a highly specific, relatively non-invasive, two-way communication channel. If this occurs, the methodology has important possibilities for neural prostheses. The goal of this project is to perform initial experiments to establish the feasibility of communication by means of a neuron probe. The tissue we have chosen in which to initially implant the probe is the rat hippocampus. If initial studies are successful, probes will be designed and implanted for communication with sensorimotor cortex.

The neuron probe we plan to fabricate will be made of micromachined silicon and will have sixteen electrodes: one conventional electrode to detect activity during placement of the probe, and fifteen within wells into which neurons will be placed. Its configuration will be similar to that of passive multielectrode probes which have been developed. It will be implanted when the cultured neurons are very young, and after a time of weeks it is hoped that they will have survived and made two-way synaptic connections. By stimulation and recording in the host and probe, we will test for the existence of such connections. An essential feature of the experiments is that the viability of the implanted neurons will be independently determined over time by stimulating them and recording their resulting action potentials.

Summary

During this quarter dummy neurochips were successfully fabricated with what we call a "canopy" structure. For these wells, the outgrowth paths for axons and dendrites are long thin channels under a silicon nitride canopy, 15 or 30 microns long, with heights of 0.2, 0.3, or 0.5 microns and widths of 2, 4, or 10 microns. In initial tests, mainly for just one week, it was observed that during the first few days a few cells did escape, most likely through the top hole. No neurons were seen in the act of escaping through tunnels. Later, no escape took place.

In these preliminary experiments, with poor statistics, good outgrowth occurred for all sizes of tunnel, even the smallest. We are optimistic that the neuron escape problem has finally been solved, and during the coming quarter experiments with good statistics, extending over longer times, will guide a final decision on canopy well parameters.

Further work has been done on visualizing DiI labelled neurons with the two-photon microscope. The background fluorescence of unstained cells has been greatly reduced by better microscope filters. The staining procedure has been refined to eliminate non-neuronal DiI fluorecence. Excellent images can now be obtained of stained neurons in wells and their outgrowing processes. Even transverse sections through the well can be made, using computer processing of the optical sections. In addition neuronal outgrowth into tissue has been very well imaged up to 150 microns deep in cultured slices.

In vivo studies have been made of probes containing stained neurons and of small injected transplants of stained neurons. After one week, stained neurons from the transplants were seen, with no outgrowth, but after two weeks, outgrowth was seen. Outgrowth of probe neurons was not observed, perhaps because the imaging was not optimal. In the coming quarter further experiments should resolve the question of whether or not probe neuron survival is a problem.

In Vitro Studies

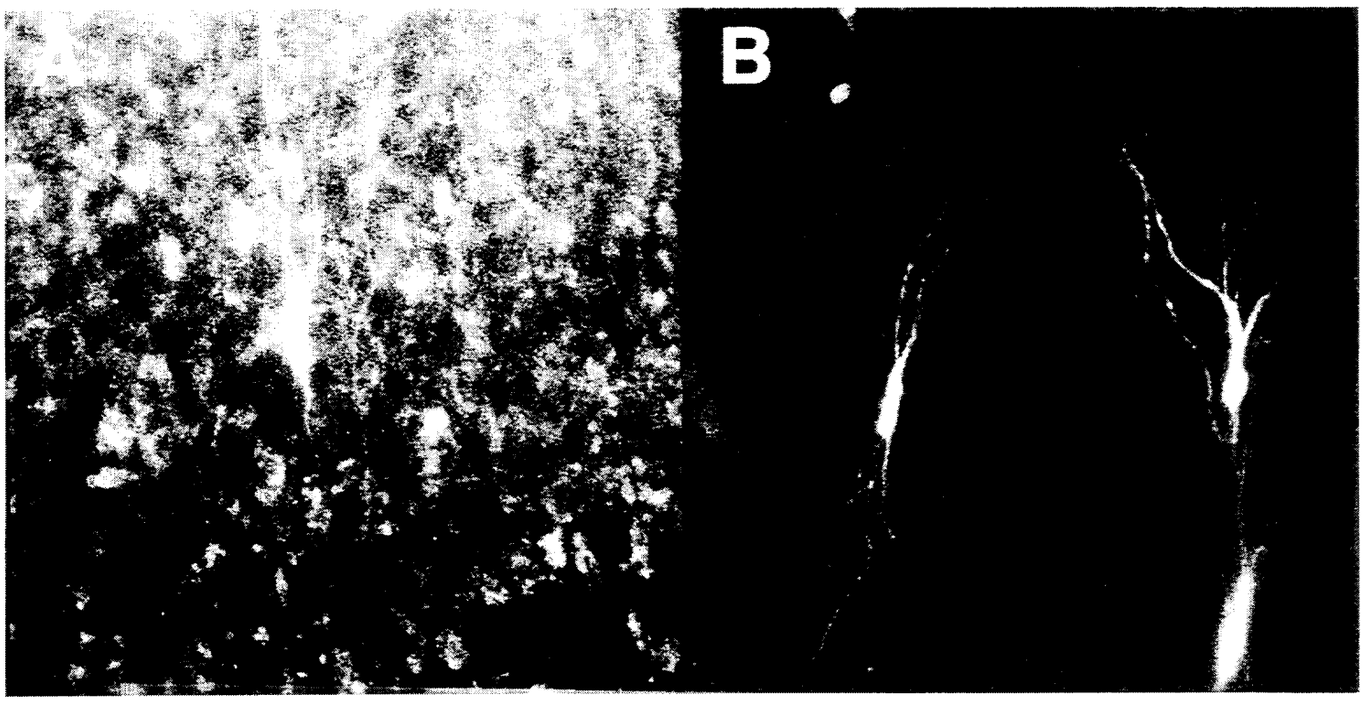
Two-photon imaging of hippocampal neurons stained with DiI

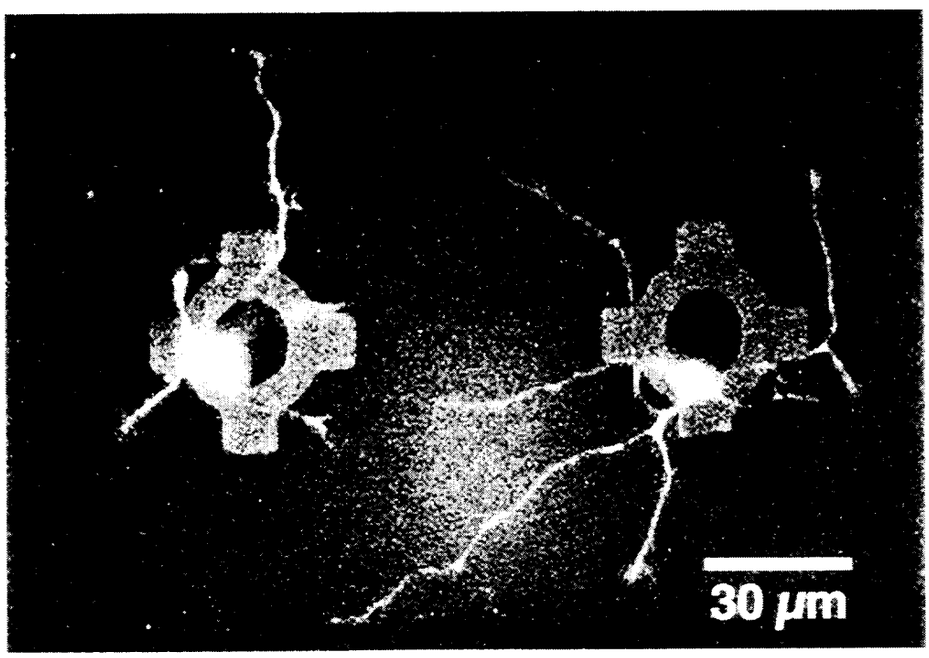
In the previous quarterly report we presented images of living neurons that were stained with DiI in suspension and transplanted to cultured hippocampal slices. We want to image the fine processes from neurons in a neuroprobe, as they grow and integrate with the host tissue. In preparation for this, we have been working on optimizing 2-photon imaging of living neurons stained with DiI. We previously used a BG39 colored-glass filter to block the infrared excitation used in our 2-photon microscope, which unfortunately blocked some of the emitted fluorescence as well. We have enhanced our signal-to-noise ratio greatly by ordering a custom dichroic short-pass mirror with a 680 nm cutoff, that can be used as an excitation filter for any of the IR wavelengths we use (700-970 nm) for any visible-emitting dye. Thus, in addition to its markedly reduced photobleaching (and thus, reduced phototoxicity), and enhanced penetration, compared to regular confocal, 2-photon microscopy has greatly relaxed criteria for filter selection, since the emission and excitation wavelengths are separated by hundreds of nm.

On the following page are shown DiI-stained neurons plated on a slice six days earlier. In six days, numerous fine processes are visible with the 2-photon microscope, extending hundreds of microns from the cell bodies, up to 150 μm into the slice. The left panel (**A**) was made using excitation at 900 nm, and the right panel (**B**), using 960 nm excitation. These are both look-through projections of 30 serial sections, 2 μm apart (60 μm total thickness). Although the laser is more powerful at 900 nm, and thus, the DiI signal is greater, we find greatly reduced background from autofluorescence at 960 nm. This autofluorescence is presumably from endogenous flavins.

We have had some difficulty with routine observations of stained neurons growing from neuroprobe wells, due to the phototoxic effects of the mercury lamp used for wide-field fluorescence microscopy. As reported in a previous update, exposures of 30 sec, commonly used for taking photographs, were lethal to the neurons. Thus, we have used the 2-photon microscope to observe

the outgrowth from the probe wells, in a non-destructive way. On the following page, neurons growing from probe wells one day after insertion are shown using Nomarski differential interference optics (on our upright Olympus microscope, top panel), and using the 2-photon laser-scanning microscope (bottom panel). The 2-photon image is a look-through projection through a stack of 10 sections at 1-μm intervals. This allows the processes as well as cell bodies to be seen. The silicon nitride grillwork is transparent but slightly autofluorescent at 900 nm, providing a handy frame of reference for determining the position of the cells in relation to the wells. Repeated scanning of these probe neurons with the 2-photon microscope had no effect on cell brightness or viability.



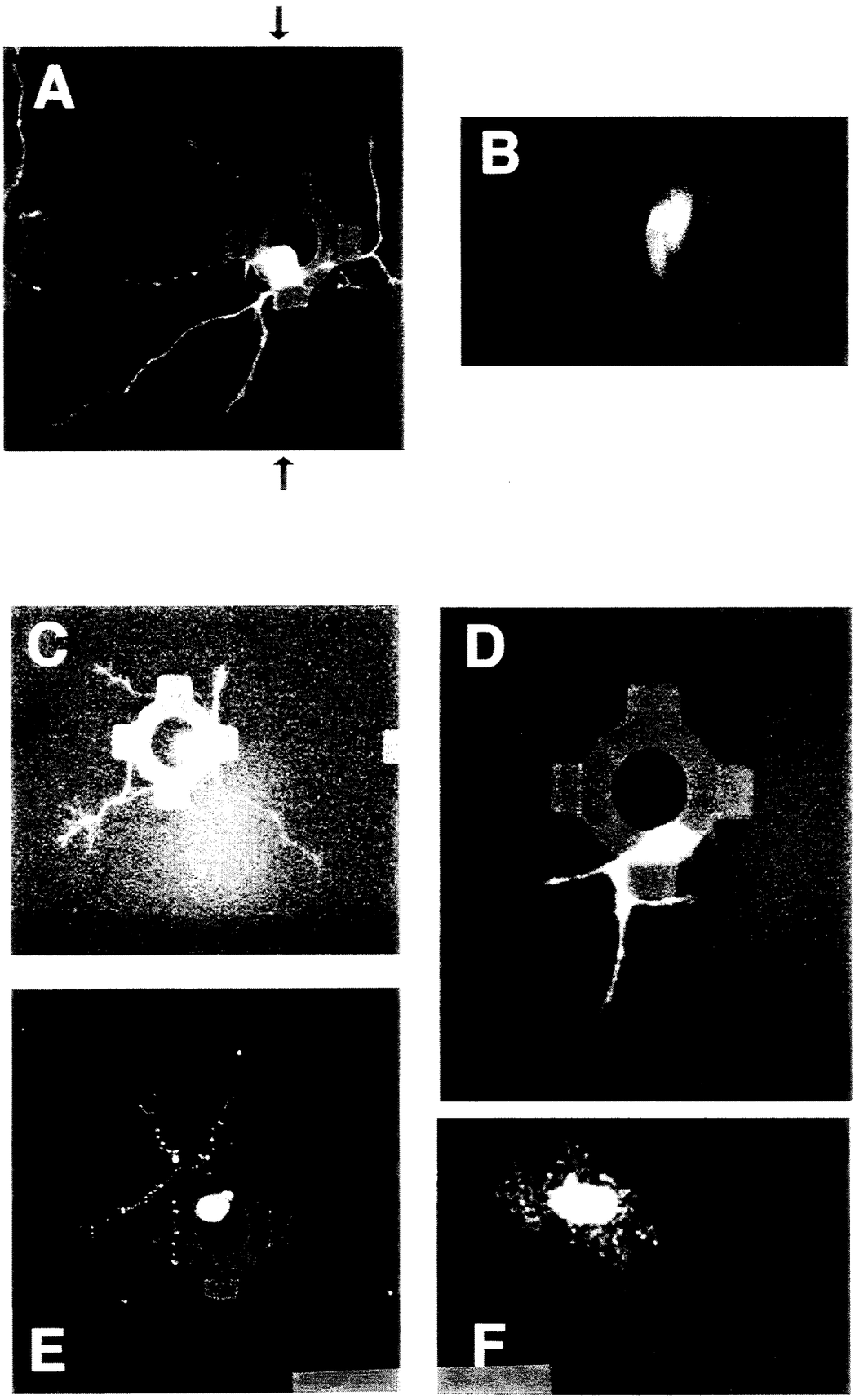

30 μm

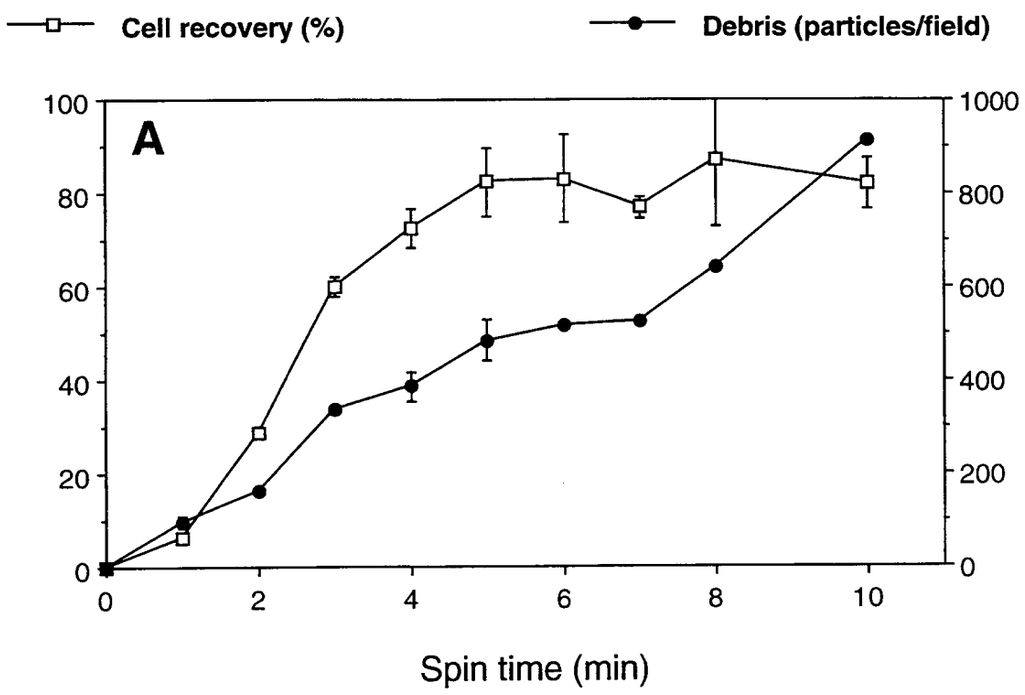
The 2-photon microscope has the additional advantage of providing information about the 3-dimensional nature of the cell shape. For example, in panel **B** on the following page the neuron in panel **A** was scanned vertically, along a line between the two arrows (utilizing the 3-d computer reconstruction capability of the two-photon system). One can see the dim signal from the grillwork, and the cell body directly beneath it, near the top of the well. Panels **C** and **D** show more neurons growing from probe wells, after one day in culture. Panel **E** shows the spotty remains of a neuron that extended processes and then died, a fate that is not uncommon; we are trying to understand why some neurons remain healthy while their neighbors die off. Panel **F** shows a glial cell that escaped from a well. The grillwork was accidentally broken off the well during cell implanting, and thus there was nothing to keep the cell inside the well. The lower-rightmost processes of the cell are touching the well, which is not visible due to the very low background staining.

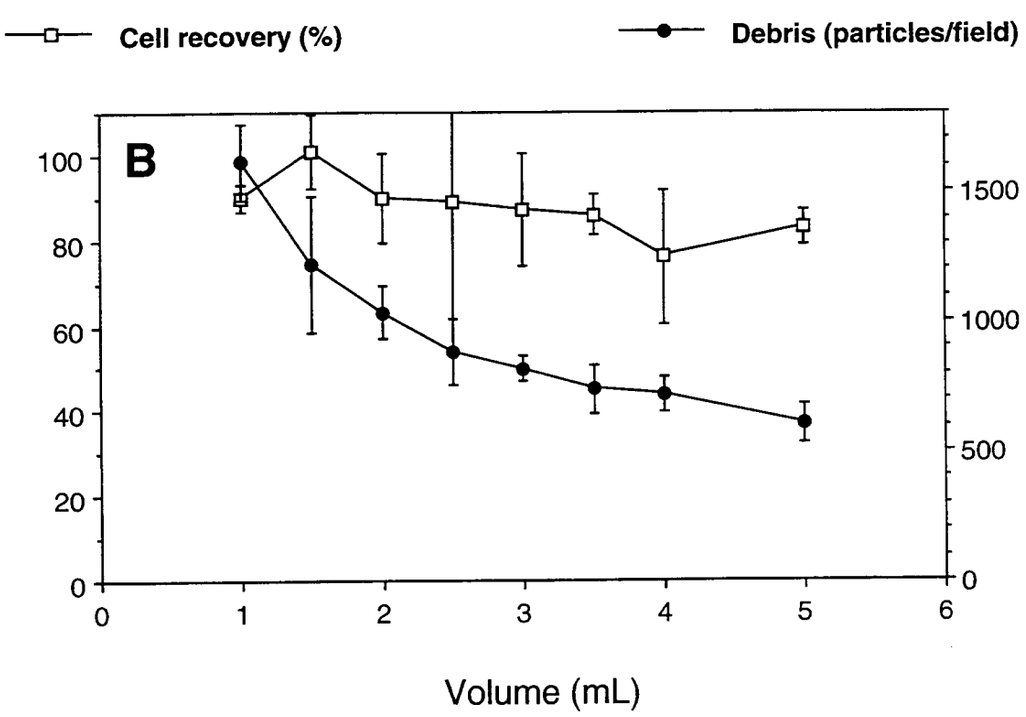
Dye and debris removal

In order to obtain such a low level of background staining, we have optimized the cell preparation and staining procedures to reduce the amount of debris present during staining, and to remove any dissolved dye. The use of the C12 form of DiI, which has a shorter alkyl tail than the commonly used C18 form, has eliminated the problem of dye crystal formation, and the inclusion of 0.0025% Pluronic F127 (a macromolecular non-ionic surfactant) in the staining medium also enhances dye solubility and transfer to the cell membranes. Centrifuging the stained cells through a layer of 5% bovine serum albumin allows almost complete removal of dissolved dye after staining.

Any stained debris present in the final cell suspension is likely to be transferred to the probe during well-filling and may cause background staining of host neurons. Therefore, we conducted a series of centrifugation studies to determine the best conditions for debris removal. Cell suspensions were prepared from neonatal rat cerebral hemispheres, including the hippocampus, and spun for various times. Pellets were resuspended and plated into petri dishes, and cells and debris were counted using a videomicroscope and NIH-Image software. The graph below shows that spins longer than 5 min (at 160 g)


A
B
C
D
E
F


Cell recovery (%)
Debris (particles/field)
A
100
80
60
40
20
0
1000
800
600
400
200
0
0
2
4
6
8
10
Spin time (min)

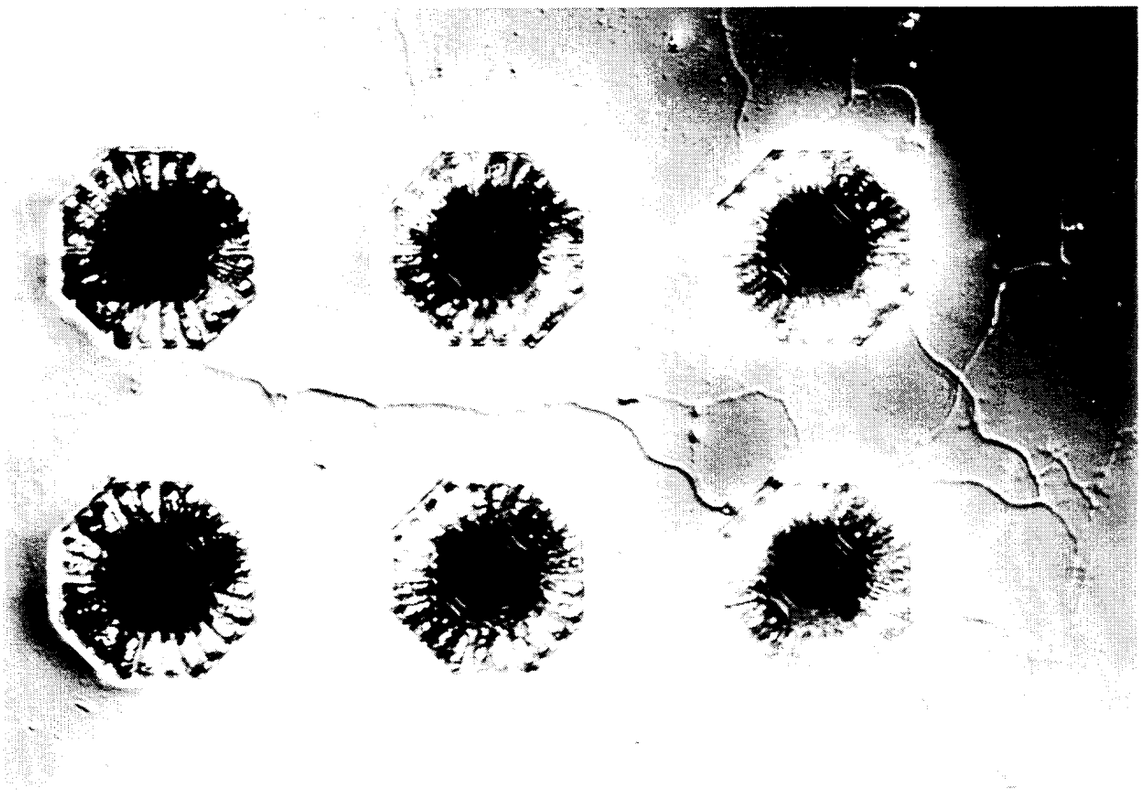

Cell recovery (%)
Debris (particles/field)
B
100
80
60
40
20
0
1500
1000
500
0
0
1
2
3
4
5
6
Volume (mL)

do not increase the cell yield, but do result in more debris. Most of the debris is still in the supernatant even after a 10 min spin, and thus there is an approximately linear increase in the amount of debris with spin time, across the range of times studied. In most cases, we have enough cells that we can afford to sacrifice many of them by using a 2-min spin, in order to produce a reasonably debris-free suspension. The lower graph shows that increasing the volume of the cell suspension (for a 6-min spin at 160 x g) has negligible effect on cell recovery, while significantly reducing the debris in the final cell pellet.

Growth of hippocampal neurons from wells

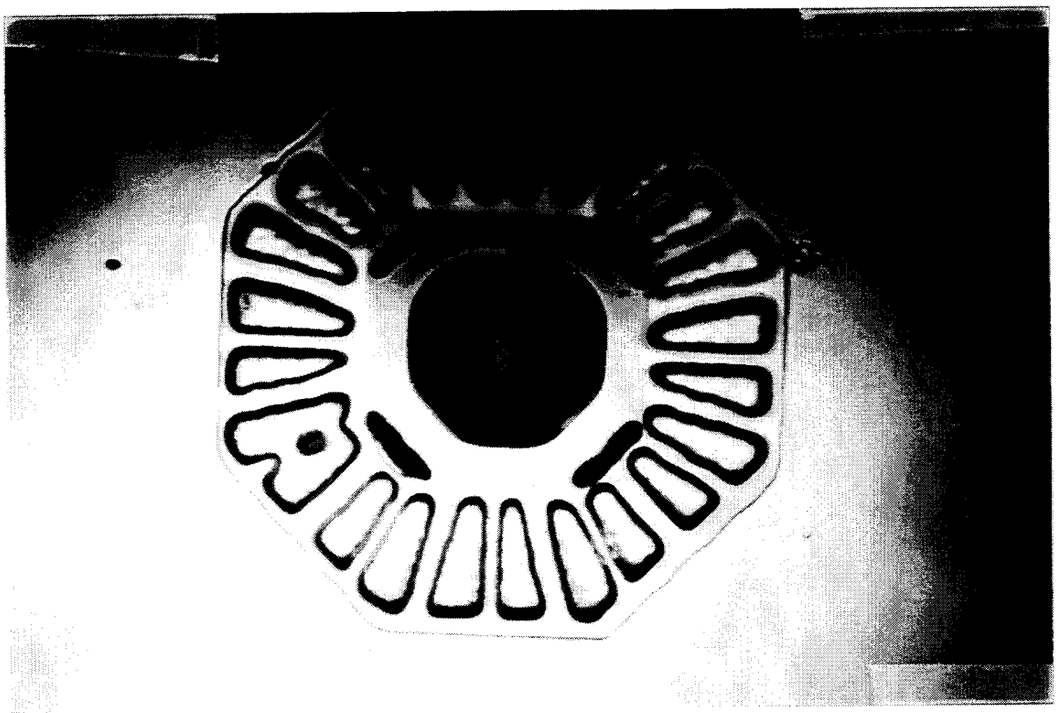
We have received a set of dummy chips (no electrodes) to test the grillwork designs described in our last QPR. In this design, the neural processes are forced to grow through a long narrow tunnel, rather than through an opening in the thin grillwork. We have previously reported that hippocampal neurons eventually squeeze through the smallest openings we are able to currently fabricate (1 by 3 μm), escaping the wells. This result is somewhat surprising, since the cytoskeleton and internal structures must undergo enormous contortions to squeeze down to this size.

The new design represents a more difficult escape path, and we hope to demonstrate that neurons will grow and remain trapped. Three criteria must be satisfied. First, the path through which the processes grow must be constrictive enough to prevent cell escape. Second, this path must not be too small, or else the growth cones will not be able to find and grow through it. Third, mature processes, which are much larger in diameter than young processes, must not be so constricted that they cannot survive. We must find the middle ground. We have several parameters to vary: tunnel cross section, minimum tunnel dimension, and tunnel length. The Nomarski photo below shows outgrowth from four of 6 of the new type wells after four days in culture. The channels are 2 x 0.5 microns, 15 microns long.



Each test chip has a total of 18 different grillwork designs, and each design has a 4 by 4 array of wells. There are two basic designs: (1) straight tunnels, and (2) staggered tunnels, with branching pathways available. Each of these has variants of 15 and 30 μm long tunnels. The tunnels were made with 1.5, 2, 4, and 10 μm widths. There is also a combined design for the straight tunnels containing all four widths of tunnel, to see if the neurons have a preferred tunnel width while growing. The minimum feature size for the photolithography is apparently 2 μm; the 1.5 μm wide tunnels did not form properly.

The tunnel height is defined by the thickness of a silicon nitride layer on the chip, which is 0.2, 0.3, and 0.5 μm on different chips, for a total of 42 different designs to test. Photomicrographs of wells with straight and staggered tunnels appear on the following page. On these photographs, the central black circle is the hole through the grillwork into the well. The surrounding square is silicon nitride overhanging the well. The dark diagonal bars across the corners are access holes through the nitride to enable the EDP etch to define the square well. The pattern radiating out from the center is the tunnels. The bottom of



20 μm

the tunnel is contiguous with the bare silicon away from the well. The top of the tunnel is defined by the nitride canopy. The areas between the tunnels are the places where the nitride makes direct contact with the underlying silicon, sealing off the tunnels from each other. The well is a full inverted pyramid, so that the bottom of the well is a point. Since there is no flat surface to reflect light, the bottom of the well cannot be seen. When chips with electrodes are made, the bottom will be flat and visible, and we will be able to image cells within the wells.

We have successfully achieved growth of hippocampal neurons from these wells. So far, we have tested 2, 4, and 10 μm wide and 0.2, 0.3, and 0.5 μm high tunnels. Initial outgrowth was rapid; several processes could be seen extending through some tunnels four hours after being placed in the wells. The transparent grillwork makes it possible to see processes before they emerge.

Freshly dissociated hippocampal neurons have been seen to have scores of fine hairs extending 10 to 15 microns from the cell bodies, which are only visible under fluorescence. They are extremely sticky, immediately attaching to any surface (even the untreated glass of the neuron pushers). These hairs can often grab onto the central hole of the grillwork when loading cells into the wells, and can sometimes allow the neuron to escape the well by migrating back through the central hole. We have noted escape by this route within minutes of loading the cell into the well. In neurochips with flat well bottoms, we can see cell bodies within the wells, and we can easily determine whether a cell is at the bottom of the well or above the grillwork. Many times, we will load a neuron into a well and see that it is resting on the bottom of the well, only to come back several minutes later to find the same cell firmly attached to the top of the grillwork with no visible processes. This type of escape can be seen up to two days after the wells are initally loaded, although it is rare once the cell has extended significant processes through the grillwork.

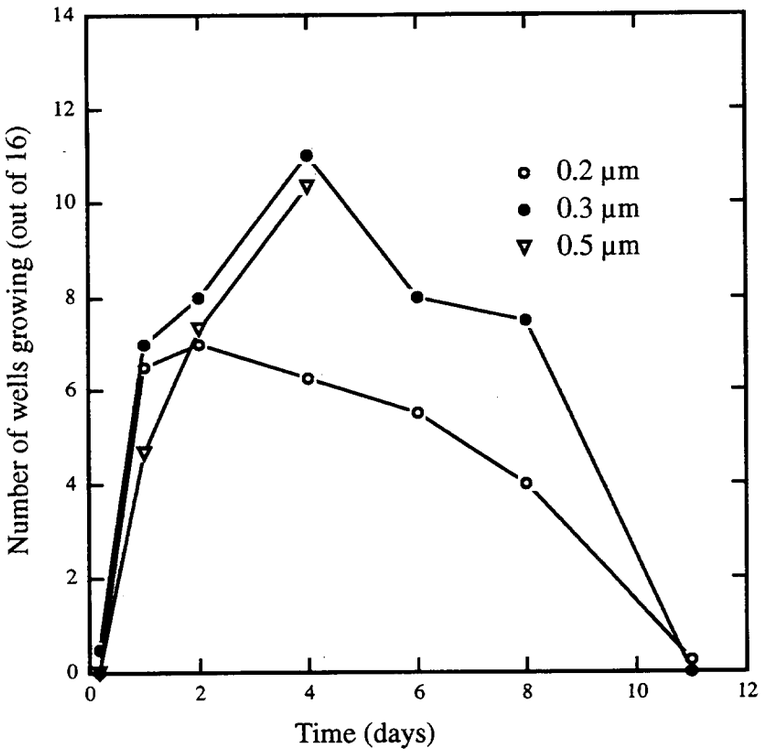
To combat loss of cells from this mechanism, we have begun to use the following method. Cell pushers with narrow (5-10 μm diameter), rounded tips are used. Cells are manipulated into the wells, either by allowing floating cells to drop in, by pushing non-adherent cells into the wells, or by allowing a cell to stick to the tip of the pusher and inserting the pusher into the well. The tip of the

pusher is then placed near the bottom of the well and gently vibrated by tapping the micromanipulator holding the pusher. This provides enough turbulence to break the cell's attachments, without damaging the cell or the grillwork. The pusher is then removed from the well before the cell has time to reattach to it.

This method greatly reduces, but does not eliminate, escape of the cells through the central holes. In these tests of grillwork containment, we are very concerned with the exact means of cell escape, and we need to be able to determine whether escape occurs through the center hole or by squeezing through the grillwork. Apparently, escape through the central hole stops 2-4 days after loading, so that after that time, any escape must come through the tunnels. Escapes within the first two days are difficult to analyze. At the moment, we can only confirm escape over the grillwork by observing the culture daily, and feeling the top of the grillwork with a pusher to see if the cell is above or below the canopy. This is not easy to do, and provides an enhanced possibility for infection. If we detect an escaped cell only after it is off the grillwork, we cannot determine how it escaped.

Below is a graph of the average number of cells growing from wells, averaged according to the tunnel height, for four chips. Since we have limited data at the moment, all different tunnel widths are averaged together. After we have tested each design type several times, we will be able to prepare a similar graph for each design, to determine which type gives the best long-term results. Several design types from four chips are shown here, all with 15 μm long tunnels: 0.2 x 2 μm^2, 0.2 x 4 μm^2, 0.2 x 10 μm^2, 0.3 x 2 μm^2, 0.3 x 4 μm^2, 0.3 x 10 μm^2, 0.5 x 2 μm^2, and 0.5 x 4 μm^2 cross-sections. The cells on two of these chips and their controls began to die off after 6 days in culture, for unknown reasons. The other two chips have only been in culture for four days as this is being written. In the coming quarter, we will obtain much more data and will be able to determine how long the cells actually survive in the new chips, and whether escape through the tunnels in indeed impossible. Up to now, we have only been able to study canopy chips with cells less than a week old.

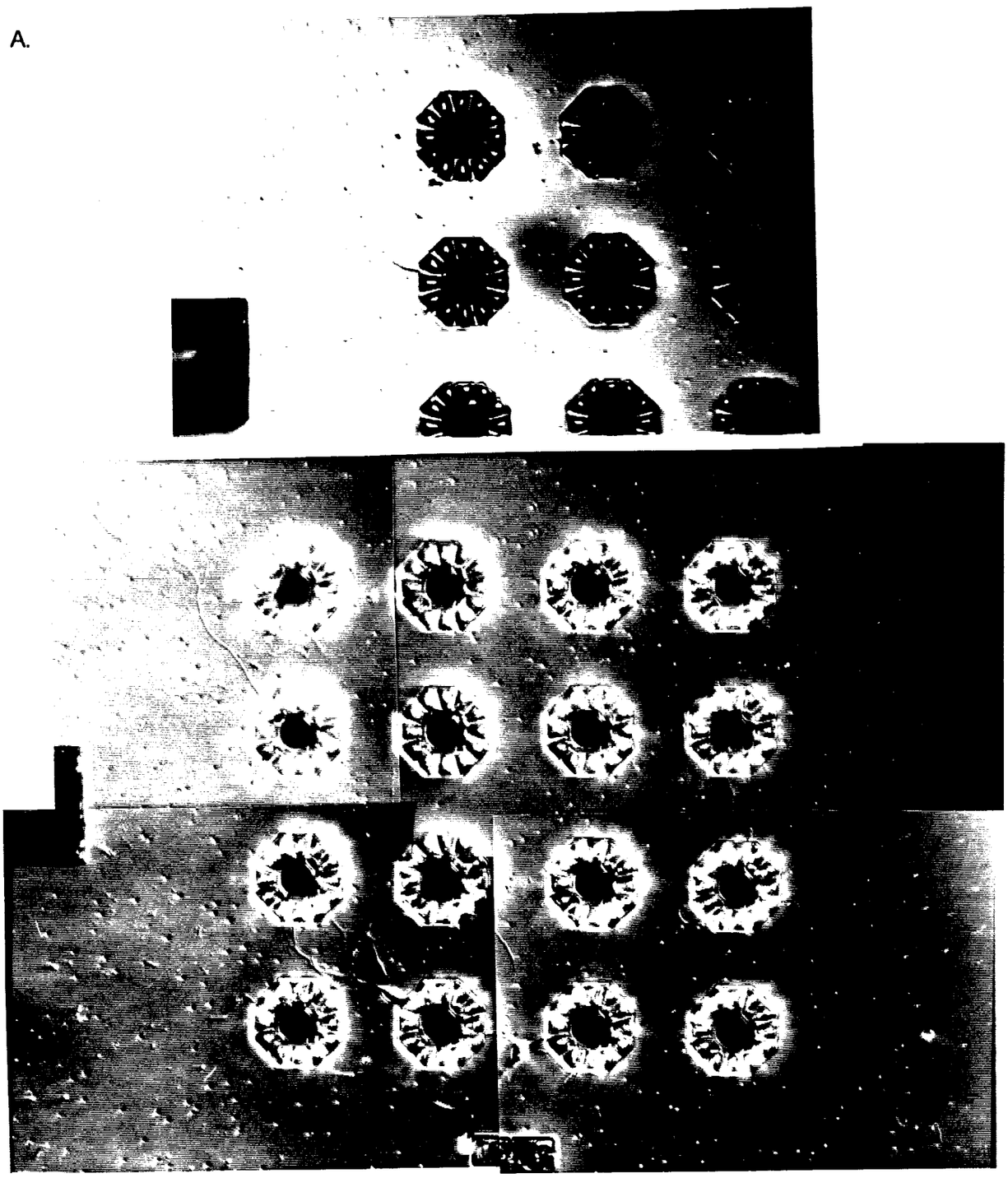
Average Growth in Canopy Chips



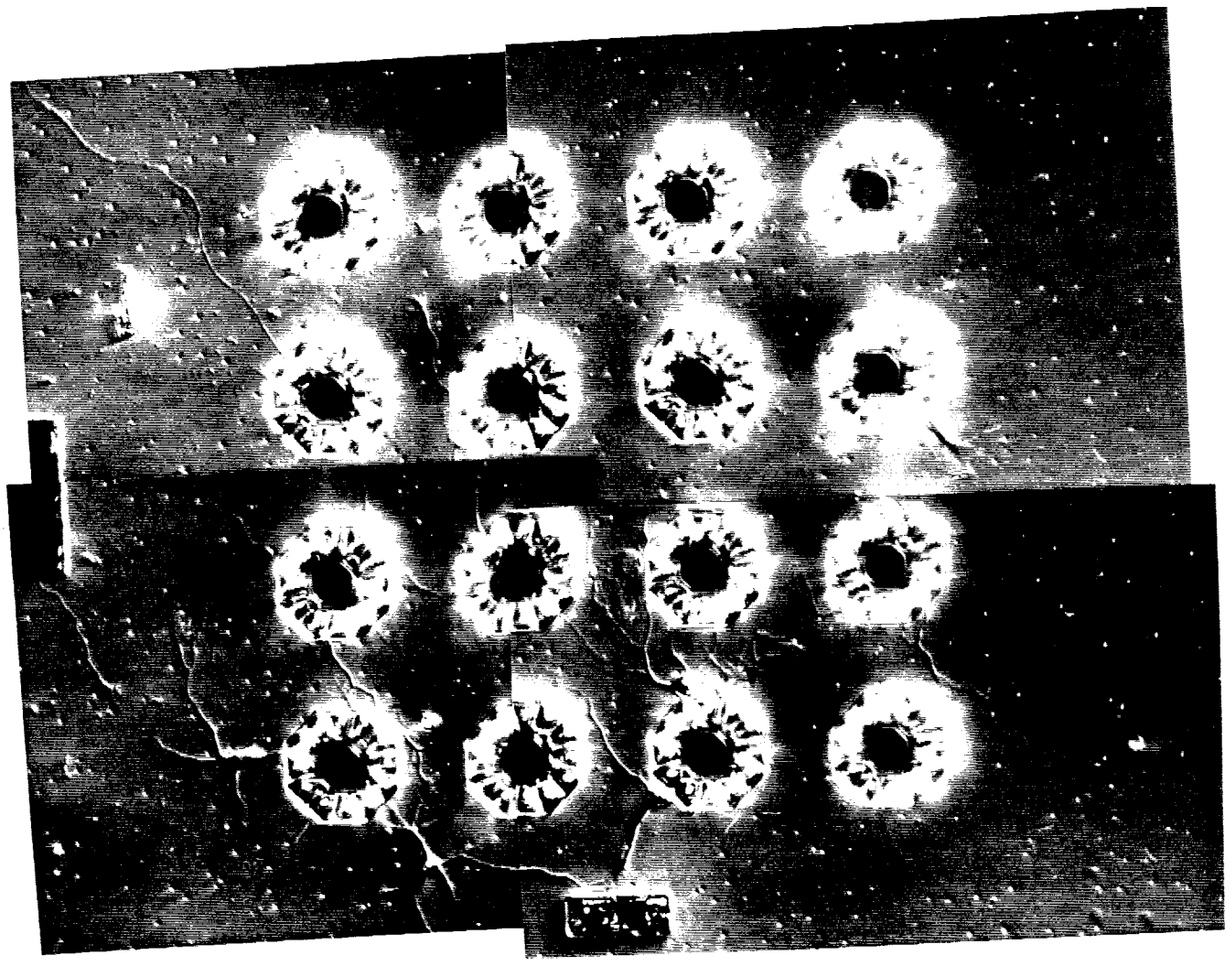
The figures on the following pages are time-series montages of the processes growing through straight 15 μm by 4 μm by 0.3 μm tunnels (chip CD10). Nomarsky images were taken at 4 hours and 1, 3, 4, and 6 days in culture. Only one process was visible at 4 hours, from well (1,2), so only that section of the chip is shown. This paticular cell grew a process in excess of 400 μm long within 24 hours, extending off the field of view of the pictures. Note the rapid development of long axons within four days, indicating that the processes are not significantly hindered by the necessity of growing through such narrow constictions. Most cells began to grow within 24 hours, but some cells required several days to begin to grow. On this chip, new processes appear from wells (1,3), (4,3), and (4,4) on days 3 and 4.

Dendrites appear much more slowly. Well (3,4) appears to have an axon and dendrites after one day, but dendrites do not emerge from any other wells until days 4 and 6. This is consistent with the growth rate of neurons in cell culture. Axons and dendrites are both present within a few hours of plating.

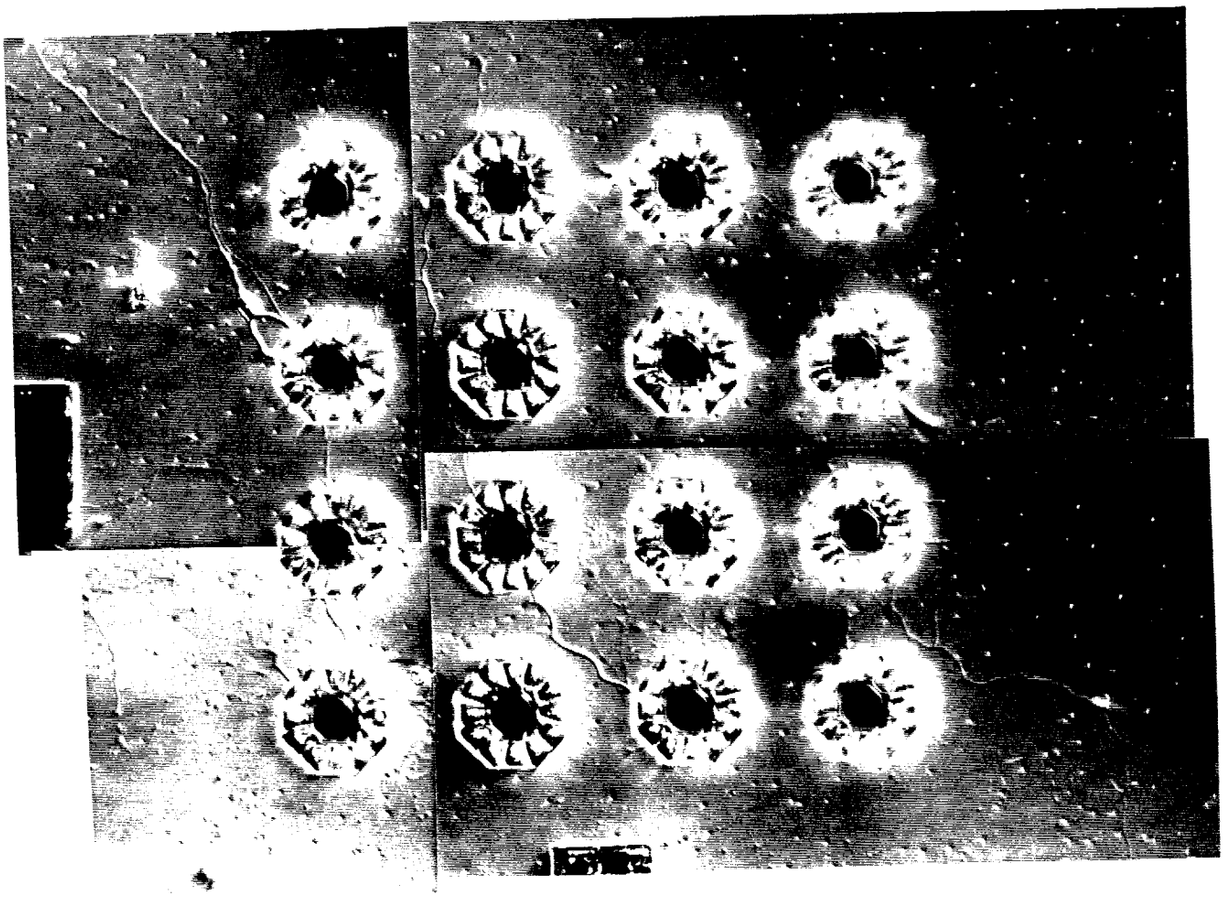
A.



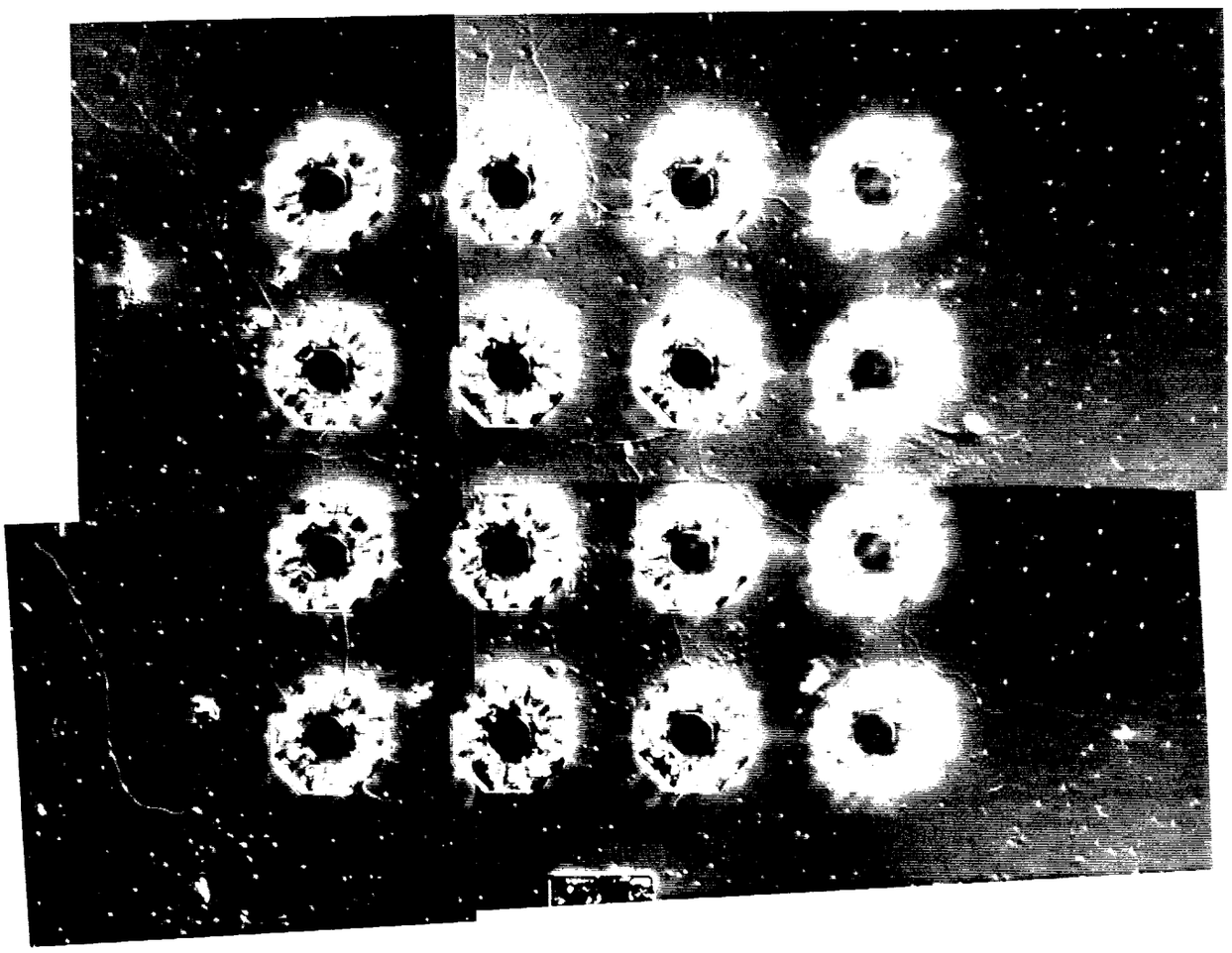
A. Growth of hippocampal neurons in chip CD10 four hours after loading wells with cells. B. Growth in CD10 after one day. Cells outside wells (4,2) and (4,3) were removed after making these photographs. On this scale, 2.6 cm = 100 μm.



Growth in CD10 after three days. Cells outside wells (4,1) and (4,3) were removed after making these photographs. The cell in well (2,4) appears to be glial; note the unusual process. On this scale, 2.6 cm = 100 μm.



Growth in CD10 after four days. Cell outside well (2,1) was removed after making these photographs. On this scale, 2.6 cm = 100 μm.



Growth in CD10 after six days. No cells appear to be outside the wells, indicating that cell escape has apparently stopped. On this scale, 2.6 cm = 100 μm.

Axons grow incredibly fast on laminin, with rates of about 50 μm/hr for the first days. However, dendrites grow at about 1 μm/hr, and a process must be about 50 μm long before it will extend out of the grillwork. So, for cells in wells dendrites are probably present in the first days in culture, but have not grown enough to be visible.

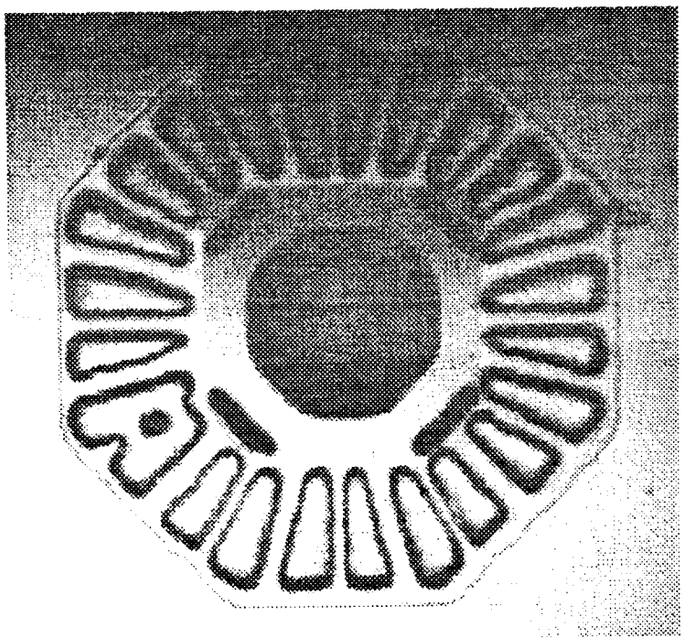
In spite of the precautions taken to prevent cells from sticking to the grillwork, escapes are still seen, some of which can be identified as coming through the central hole. For this chip, after cells were seen to be outside of a well, they were normally removed with a cell pusher to prevent their processes from being confused with those from cells still in wells. For CD10, two cells were outside the grillwork at 24 hours and were removed after taking photographs. After 3 days, four more cells were outside the wells, and were removed. After 4 days, one more cell was outside the wells. We were unable to determine the method of escape for any of these cells. There were still 10 cells growing processes out of the wells after 4 days, so that if escape does occur through the tunnels, it must be relatively slow.

From these preliminary results, it is clear that the early growth stages of hippocampal neurons are not significantly hindered by even the smallest tunnels, 0.2 x 2 μm^2. We have also not yet seen escape from the wells which we could positively identify as coming through the tunnels. However, we must grow many more cultures to determine the optimal geometry, and we still need to obtain 2-4 week old cultures in the wells.

Microfabrication

As noted in the last quarterly report, a batch of wafers had been started in a newly designed fabrication procedure. The procedure aimed to create channels rather than holes through which neuron processes will exit the wells. We deemed this necessary as the number of neurons that continue to escape from the wells is too great. During the past quarter, these wafers have been brought to the end of the process and testing has begun. Below is a photomicrograph of a finished well with 2 μm wide channels.

Microscope Photo of Canopy Well with 2.0 μm Wide Channels

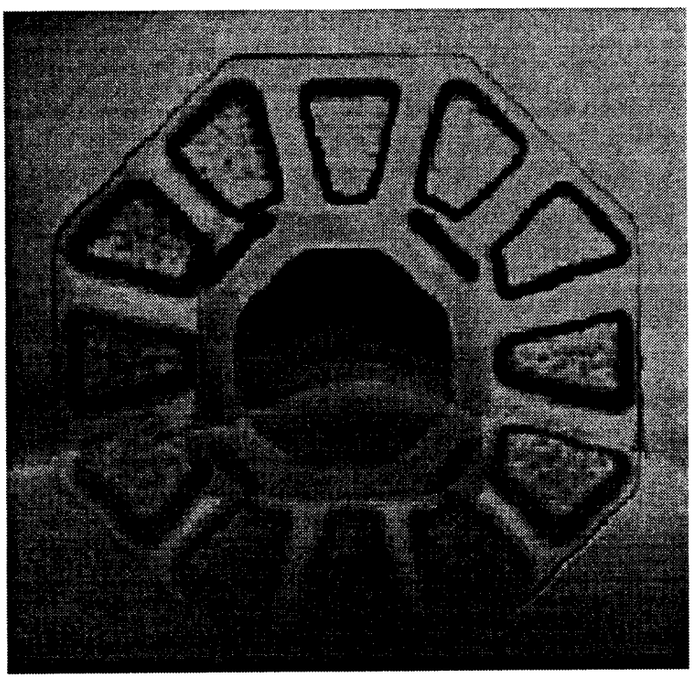


The features, as seen in the photo are as follows. The dark central octagon is the main access hole to the neuron well. It is through this hole that neurons are implanted into the chip. Immediately surrounding this hole are four smaller slots oriented at 45°. The purpose of these are to allow EDP access to the silicon beneath permitting the correct size well to be formed. If not present, the dimensions of the well would be constrained to the size of the center

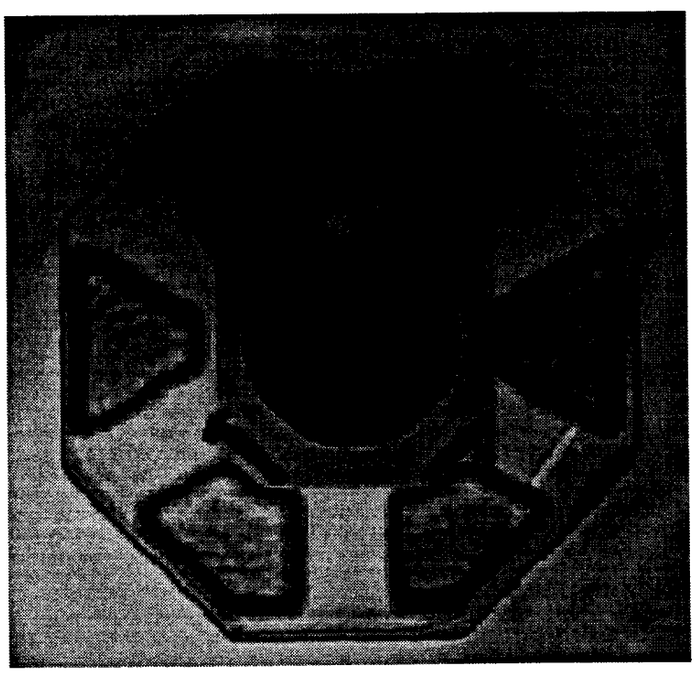
octagon. Moving out further from the center of the well is a ring of trapezoidally shaped features. These are the posts that anchor the nitride canopy to the silicon substrate. They are created by etching down through an LTO spacer layer and into the silicon below. It is the RIE etching that forms these holes that roughens the trapezoidal areas as seen in the photos. The final features in the photos are the lanes that separate the trapezoids. These are the channels through which neurons will extend their processes. Each channel consists simply of a nitride canopy raised above the silicon substrate. The following page shows canopy wells with wider channels.

At present, microfabrication is on hold. Neurons are being stuffed in the set of canopy chips produced and studies are being conducted to determine which dimensions are best at containing the cells. Once height, length and width for the channels are decided upon, we will embark upon creating a batch of real neurochips for *in vitro* studies and also canopy well dummy probes for *in vivo* studies.

Microscope Photo of Canopy Well with 4.0 μm Wide Channels



Microscope Photo of Canopy Well with 10.0 μm Wide Channels



In Vivo Studies

During this period the main efforts were directed to obtain reliable DiI stained hippocampal neurons in culture using the new type of silicon probes and to transplant them in the hippocampus of the adult rats. A new DiI staining protocol (described in previous report) was used for visualization of neuronal outgrowth.

Of the 34 loaded silicon probes, initial process outgrowth was observed in eighteen probes, as summarized in the table below.

No. of rat.	What was transplanted	Number of cells	Days of survival	Result
R118dp	DP38 left hip DP35 right hip	5 cells within wells, 18 on a surface	7	No fluorescent processes within host brain
R119sus	6 injections of DiI stained cells	300-800 cells in each injection	7	Fluorescent cells within transplant, no outgrowth
R120dp	DP26 left hip DP46 right hip	6 cells within wells, 14 on a surface, all with processes	7	No fluorescent processes within brain.
R121dp	DP42 le. hip rostral DP29 le. hip caudal DP25 ri. hip rostral DP27 ri. hip caudal	1 cell within well 3 cells within wells 2 cells within wells 4 cells on a surface	7	No fluorescent processes within host brain

R122dp	DP52right hip DP48 left hip	10cells within wells 8 cells within wells	30	No fluorescent processes within host brain.
R123dp	DP44 righthip DP51 left hip	6 cells within wells 8 cells within wells	30	No outgrowth of processes in a host brain were found
R124sus	6 injections of DiI stained cells	200-800 cells in each injection	9	There are living cells within transplant, no processes yet.
R125sus	6 injections of DiI stained cells	200-800 cells in each injection	18	Outgrowth axons and dendrites from transplant into host hipp.
R127sus	6 injections of DiI stained cells	200-800 cells in each injection	17	Outgrowth axons and dendrites from transplant into host hipp.
R129dp	DP28left hip DP43 right hip	16 cells within wells 16 cells within wells	18	Cell bodies of two cells were found within wells, but no outgrowth of processes.

These probes were transplanted into the hippocampus of seven rats. Two types of control procedures were used to examine the problems associated with cell survival in the grafted probes. First, the handle areas of the probes were placed back in Petri dishes and culturing was continued. Several of the probe handles got infected by fungi likely during the transplantation procedure. In the remaining cases, neurons and glia with processes were observed on the surface of handle all the way to the broken the surface which was in contact with the brain during the grafting procedure. These controls provided evidence that neurons may continue growing even after the grafting procedure.

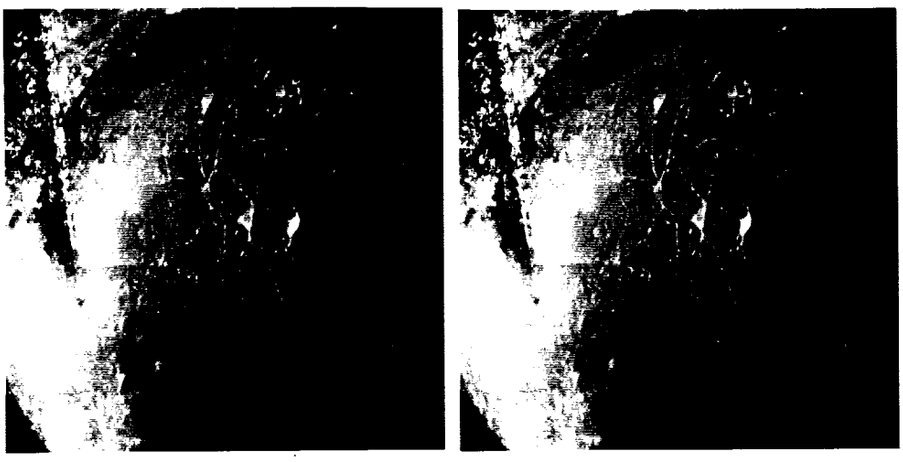
Second, at the end of the loading and grafting procedure the remaining neurons in the suspension were used for grafting without loading them into the probes. Very small suspension grafts were placed at 4 to 6 sites in 5 rats. The volume of injected cell suspension varied between 0.5-1.0 μl, and cell density between 300-800 cells per microliter.

One rat with cell suspension grafts and four rats with silicon probes were perfused one week after transplantation and process outgrowth was examined with the 2-photon laser-scanning microscope. No outgrowth of neuronal processes was observed from the ten probes or from cell suspensions transplants at this time point. Disappointingly, no evidence of cell survival was obtained with this method. A caveat is that the 2-photon method is capable of examining less than 200 μm of tissue. On the other hand, the brains were cut at 400 μm sections to assure that the probe was cut together with the brain.

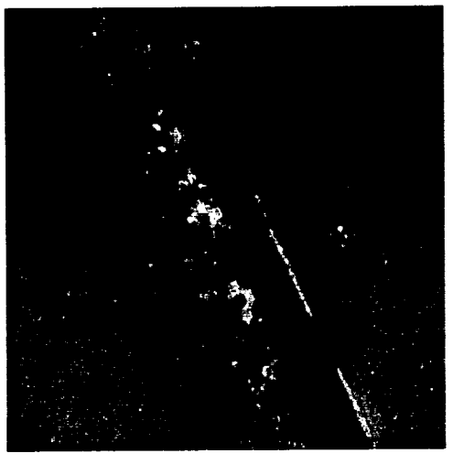
The figure following illustrates some results. The top two panels show a stereo pair (from a section series) of an implant after 7 days, with the inserted probe visible to the left, surrounded by a large amount of fluorescent debris, and no obvious outgrowth from the wells. Some of the host pyramidal neurons took up the dye, and their fine processes can be seen extending several hundred μm from the cell bodies. In the middle and lower images, another probe is visible, after 18 days, with greatly reduced debris due to improvements in cell preparation and staining. The grillwork of several wells is visible, but no outgrowth from the wells was observed. The bottom panels show horizontal (left) and vertical (right) sections through a well that contained the remains of a dead cell.

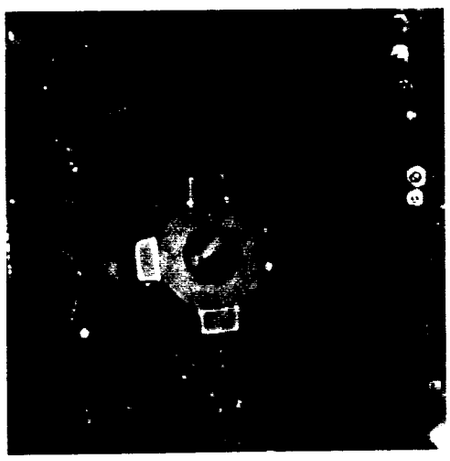
Outgrowth from cell suspension, however, was observed in rats two-three weeks after transplantation using conventional fluorescence microscopy. Cells developed dendrites and axons which grew into the host brain. The second page of pictures below shows an example with several neurons growing on the surface of the thalamus fourteen days after transplantation. The top left and top right views are with bright field and fluorescence microscopy; the bottom view shows details of dendritic outgrowth.

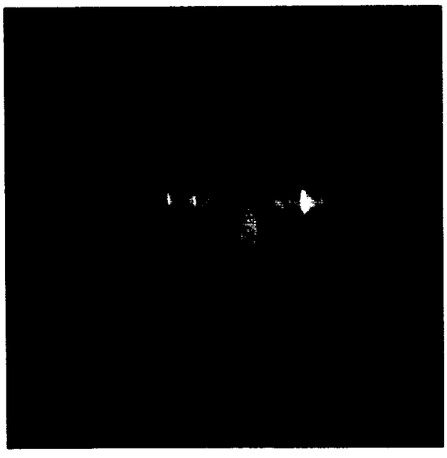
R121DP Rhip1

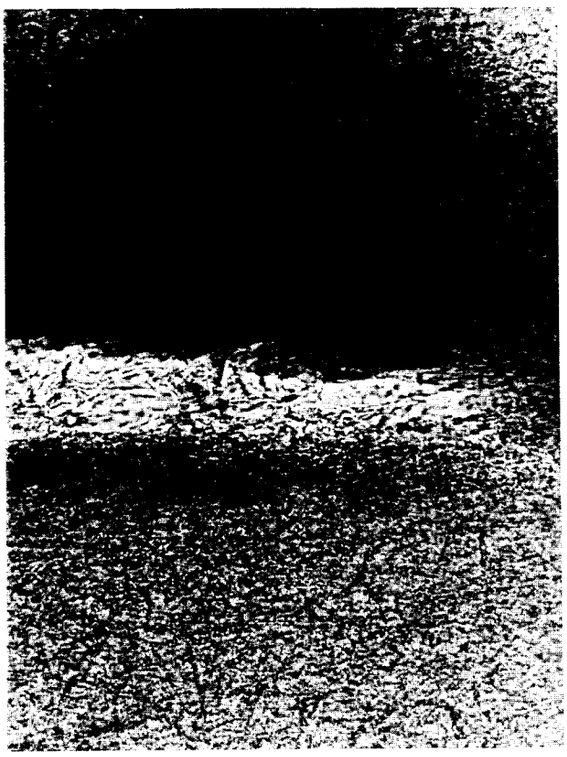


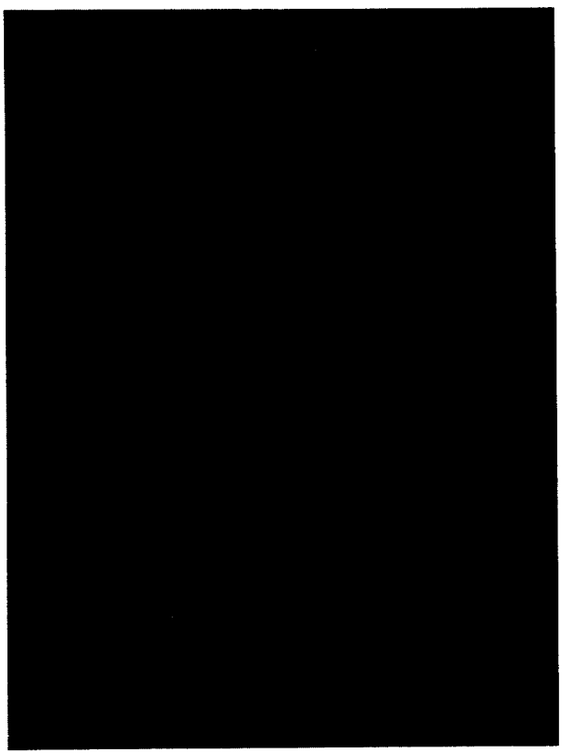
129DPRight

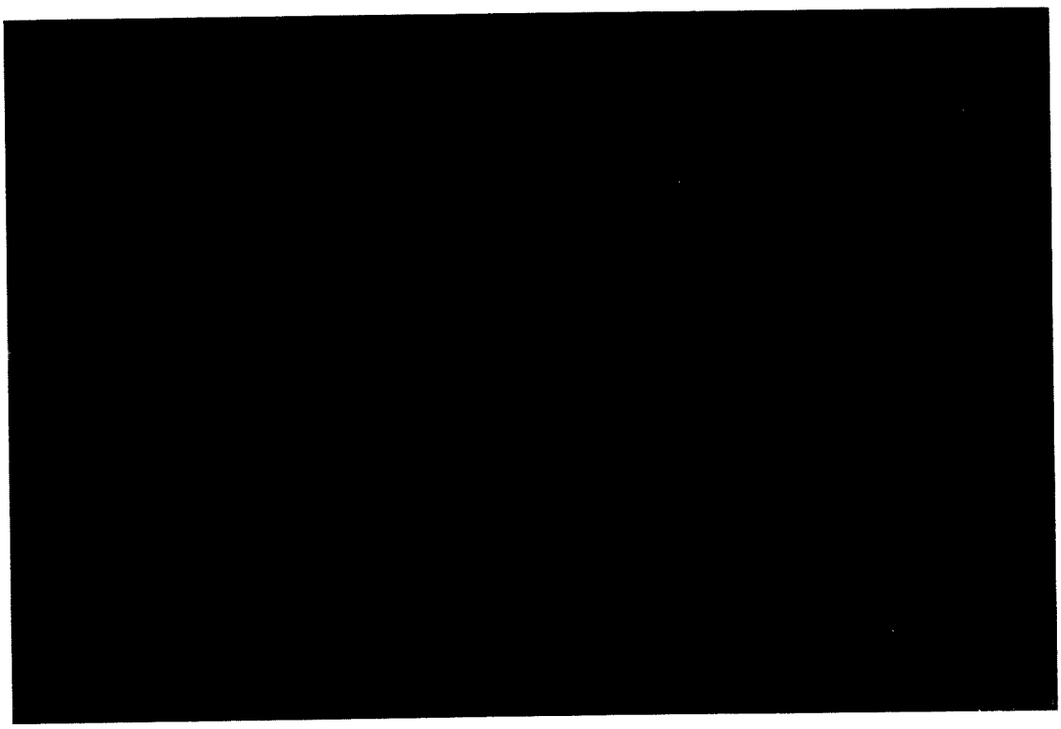












Several small cells were observed within transplants and at the border with the host brain. The success of the staining procedure during loading was clearly demonstrated by the fact that no dye debris was observed in the tissue culture before transplantation. However, *in vivo* small round fluorescent particles were often observed. Comparison of fluorescence and Nissl stained sections suggested that DiI was accumulated in microglia. It is likely that some of the grafted neurons died and they were phagocytosed by microglia along with the DiI particles.